# A HIGHLY SENSITIVE AND SPECIFIC ENZYME-IMMUNOASSAY METHOD FOR OESTRADIOL-17β

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## 1. Introduction

Consideration indicates that enzyme-immunoassay could overcome the serious drawbacks of expense and radiation hazard inherent in the radioimmunoassay technique [1–4]. Unfortunately despite several efforts over the past few years, developed enzyme-immunoassay procedures have not as yet seriously rivalled the popular established radioimmunoassay method on both counts of sensitivity and specificity.

The development of a highly sensitive enzyme-immunoassay method for measurement of hormones such as oestrogens at the sub-picomole level, demands a critical choice of high specific activity enzyme [5] which has to be stable on chemical modification and the measurement of its activity must be simple and sensitive. Horse radish peroxidase (EC 1.11.1.7) which appears to satisfy most of these requirements has been used for the enzyme immunoassay of oestradiol-17β [6–8]. *Escherichia coli* β-D-galactoside-galactohydrolyase (EC 3.2.1.23) also possesses the above required properties: however its high specific activity makes it superior to peroxidase. Although several antigens have been conjugated to this enzyme and used in enzyme-immunoassay procedures [9–11], as yet no enzyme immunoassay method using the enzyme for the determination of oestradiol-17β has been reported. The preparation and affinity chromatography purification procedures of an *E. coli* galactosidase-oestradiol-17β conjugate in [12] were shown to be of paramount importance for obtaining pure enzyme–steroid label for highly sensitive enzyme-immunoassay.

Improvement and simplification of these previous preparation and purification procedures, coupled with the use of the double antibody precipitation technique and a judicious choice of heterology between antiserum and enzyme–steroid conjugation bridge [7] has led to the development of a simple highly sensitive and highly specific enzyme-immunoassay method for oestradiol-17β. This method is as simple, sensitive and specific as the radioimmunoassay technique for this hormone, and possesses the additional advantages of lower cost and freedom from radiation hazard.

## 2. Materials and methods

### 2.1. *Reagents*

[2,4,6,7-$^3$H]Oestradiol-17β (spec. act. 85 Ci/mM) was obtained from the Radiochemical Centre, Amersham and non-radioactive steroids from Koch-Light, Colnbrook, Bucks. 1-Cyclohexyl-3-(2 morpholinoethyl) carbodiimide metho-*p*-toluene sulphonate (CMC) was obtained from Sigma, St Louis, MO; Sepharose 4B, DEAE–Sephadex A-50 and Sephadex G-25 were obtained from Pharmacia, Uppsala. The synthetic substrate *o*-nitrophenyl-β-D-galactoside (ONP-G) and mercaptoethanol were obtained from the British Drug Houses, Poole, Dorset. The hapten used for enzyme conjugation, oestradiol-17β-6-(*O*-carboxymethyl)-oxime ($E_2$6CMO) was synthesised according to [13]. Antiserum to oestradiol-17β raised by oestradiol-17β-3 hemisuccinate-BSA was prepared according to [14]. The assay buffer used for both enzyme and radioimmunoassay was 0.1 M phosphate buffer (pH 7.0) 0.01 M $MgCl_2$, 0.1 M NaCl, 0.1%

sodium azide and 0.1% gelatin. Donkey anti-rabbit serum was obtained from Burroughs Wellcome, Beckenham. An SP6 spectrophotometer (Pye Unicam, Cambridge) was used for absorptiometric measurement.

2.2. *Preparation of the enzyme–steroid conjugate*

The steps involved in the preparation and the purification of the conjugates have been described [12]. Modifications, however, have been introduced to these procedures. The first step was to obtain and purify β-D-galactosidase from *E. coli.* Mutant K12 3300 cells were cultured and harvested in the normal way, and then the cells ruptured on a French press machine. The resultant suspension was centrifuged to remove insoluble material before subjection to 40% ammonium sulphate precipitation. The crude enzyme was then purified by DEAE–Sephadex-A50 [15] to a high specific activity to ONP-G substrate (350 μM/mg/min). The enzyme–steroid conjugate was prepared using $E_2$6CMO. Condensation of this compound with lysyl groups of the enzyme to form a peptide link was effected using the carbodiimide CMC. An intermediate of $E_2$6CMO and CMC was first formed in 0.2 M phosphate buffer (pH 6.70, containing 0.1 M NaCl) by reacting 1 mg $E_2$6CMO (0.5 mg/ml) with 84 mg CMC added in solid form at 10–15°C for 30 min. This intermediate was then added slowly to 10 mg enzyme (20 mg/ml buffer) and the mixture stirred at 4°C for 16 h. This conjugation procedure was milder than the method in [12] and no loss of specific activity of the enzyme occurred during the conjugation step. The enzyme conjugate was purified by dialysis, gel-filtration and a steroid affinity column step as in [12]. The enzyme affinity column step was not needed, since as stated the mild conjugation had preserved the specific activity of the enzyme. An aliquot of the final purified *E. coli* β-D-galactosidase–oestradiol-17β conjugate was hydrolysed with acid and the amount of 6-keto-oestradiol-17β produced determined by radioimmunoassay; this enabled assessment of the enzyme/steroid ratio which was found to be 1:1.7.

2.3. *The enzyme-immunoassay method*

$E_2$3HS antiserum, 0.1 ml (dilution 1:3200; $3.6 \times 10^{-10}$ mol/litre anti-$E_2$ activity), 0.1 ml enzyme–steroid conjugate (82 ng/ml) and 0.1 ml various doses of $E_2$ standard were incubated at 37°C for 1 h. The mixture was then allowed to cool for 20 min, then 0.1 ml donkey anti-rabbit antiserum (dilution 1:25) added. Incubation was continued for 16 h at 4°C. The immune precipitate was washed twice by the addition of 1.0 ml assay buffer then centrifuged at 3000 × *g* for 20 min in a refrigerated centrifuge; the supernatant was then decanted. The enzyme activity in the immune precipitates was measured by adding 0.2 ml ONP-G substrate (0.7 mg/ml in assay buffer containing 0.1 M mercaptoethanol) and incubating for 20 min at 37°C in a thermostatically-controlled waterbath. The reaction was stopped by the addition of 1 ml 1.0 M sodium carbonate solution. The amount of hydrolysed substrate was estimated by measuring $A_{405\ nm}$.

A radioimmunoassay method was set up using exactly the same conditions as above except that the enzyme label was replaced by its calculated equivalent of 12.7 pg [2,4,6,7-$^3$H]oestradiol-17β and the performance of both immunoassays was compared.

## 3. Results

A comparison between typical enzyme-immunoassay and radioimmunoassay standard curves for $E_2$ is shown in fig.1. The results show that under the conditions described, the enzyme-immunoassay method was slightly more sensitive than radioimmunoassay. The specificity of the respective immunoassay techniques was assessed by both the well-established technique in [16] and by a recently-devised logit–log system [17]. Both methods of assessment made for the three related ligands, oestrone ($E_1$), oestriol ($E_3$) and oestradiol-17α ($E_2\alpha$) showed that the enzyme-immunoassay method tended to be slightly more specific than radioimmunoassay. Defining the cross reactivity of the standard ligand $E_2$ as 100% for both methods, the enzyme-immunoassay results were $E_1$ 0.7%, $E_3 < 0.1\%$, $E_2\alpha$ 0.4% and for radioimmunoassay were $E_1$ 1.5%, $E_3 < 0.1\%$ and $E_2\alpha$ 0.4%.

Various doses of $E_2$ ranging from 50–500 pg were added to human male plasma (of known $E_2$ content) extracted with ether, and assayed by both immunoassay procedures. Almost identical results were obtained from these methods indicating that the enzyme-immunoassay technique can be used to assay human plasma samples.

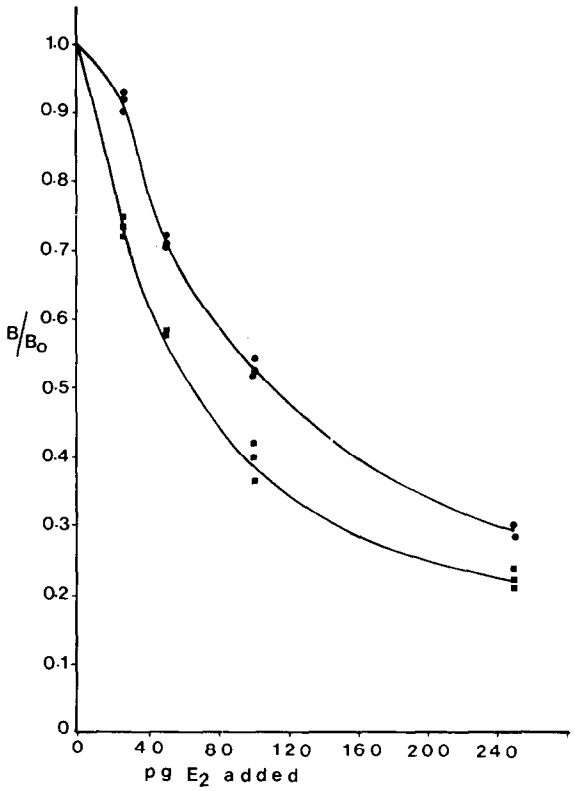


Fig.1. Standard curves showing amount (%) bound enzyme activity for various oestradiol-17β doses ($B$) as a ratio with that bound using the enzyme-labelled conjugate alone ($B_0$); compared with similar results for radioimmunoassay. (•) Radioimmunoassay. (■) Enzyme-radioimmunoassay.

## 4. Discussion

The described oestradiol-17β method involves probably the simplest, most sensitive and specific enzyme-immunoassay technique yet produced for any compound. The high sensitivity of the enzyme-immunoassay method can be mainly attributed to the use of the high specific activity enzyme and the preparation and purification of the low molar ratio enzyme–steroid conjugate used as immunoassay label. Factors involved in the preparation and purification procedures which helped to preserve the enzyme activity of the conjugate and therefore the sensitivity of the immunoassay are:

(i) The mild enzyme–steroid conjugation technique which did not cause any loss of enzyme activity;

(ii) The affinity chromatography purification step, which still preserved this activity.

Again the affinity chromatography step, removed all unconjugated enzyme and thus eliminated any background enzyme activity which if not removed would interfere with enzyme immunoassay by increasing the blank, hence lowering the sensitivity. Another factor in the enzyme-immunoassay method affecting sensitivity is the use of the double antibody precipitation separation procedure which unlike solid phase methods is not only simple, but the immune precipitate does not interfere with enzyme activity.

Alternative assessment of the *E. coli* β-D-galactosidase activity by the more sensitive fluorogenic substrate 4-methyl umbelliferyl β-D-galactoside was found to be too sensitive for the enzyme-immunoassay endpoint determination. Since the limit of sensitivity of any immunoassay method is dictated by the apparent equilibrium constant ($K_{app}$) of the heterogenous antibody collection with the antigen or hapten [18], this means that use of higher $K_{app}$ antiserum should enable determination of oestradiol-17β at sub-picogram levels. This is one of the rare occasions when the measurement of the labelled tracer has been recorded as being too sensitive for immunoassay at picogram levels.

Previous heterologous enzyme-immunoassay methods [7] have invariably sacrificed specificity at the expense of sensitivity. The heterologous combination between the specific $E_2$3HS antiserum [14] and the enzyme–$E_2$6CMO conjugate produced specific as well as sensitive enzyme-immunoassay, because the *O*-carboxymethyl-oxime bridge link between enzyme and steroid was not antigenically recognised by the antiserum.

The simple automatable colorimetric measurement of enzyme activity using a standard laboratory spectrophotometer and an inexpensive readily available substrate makes the enzyme-immunoassay method cheaper and possibly simpler than radioimmunoassay. These desirable advantages coupled with the fact that the enzyme-immunoassay method is just as sensitive and specific as radioimmunoassay methods for oestradiol-17β and even possesses a potential sensitivity superior to these latter techniques, as well as freedom from radiation hazard using an enzyme label superior to radioactive labels as regards shelf

life; all makes the enzyme immunoassay method more than a serious rival to radioimmunoassay and may become the technique of choice in the future.

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